Effects of Long-Term Storage on Potency of TM Biocontrol-1, the Registered Viral Insecticide of *Orgyia pseudotsugata* (Lepidoptera: Lymantriidae)

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ABSTRACT The article presents the results of bioassaying 39 samples of TM Biocontrol-1, a viral insecticide, from 10 different lots and various sizes of vacuum-sealed packages that were stored at −10°C for 5–15 yr. This is the first study to present potency data for a registered virus product stored for this length of time. Laboratory bioassays in insects from the same colony from which the TM Biocontrol-1 was produced showed that the stored viral insecticide is still effective, although it lost ≈30% of its effectiveness during storage. A direct correlation of this loss with the length of time in storage is not apparent. Bioassays also showed that there are significant differences in the susceptibility of Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough), larvae from different geographic regions to *Op*MNPV (family *Baculoviridae*, genus *Nucleopolyhedrovirus*) infection. Package size did not affect the potency of stored TM Biocontrol-1. There were no clear, significant differences in the effectiveness among lots of TM Biocontrol-1 processed by different organizations.

KEY WORDS *Orgyia pseudotsugata*, nucleopolyhedrovirus, TM Biocontrol-1, storage, efficacy

TM BIOCONTROL-1 IS A VIRAL insecticide produced by the United States Department of Agriculture–Forest Service (USDA–FS). It was registered in 1976 by the U.S. Environmental Protection Agency for the control of Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough) (Lepidoptera: Lymantriidae), a cyclic forest defoliator in the Pacific Northwest. Between 1985 and 1995, the USDA Forest Service produced and stored TM Biocontrol-1 sufficient to treat >400,000 acres. TM Biocontrol-1 was produced in the Goose Lake laboratory strain of *O. pseudotsugata* at the USDA–FS facility in Corvallis, OR (Martignoni 1978, 1999; Hadfield and Magelssen 1995). This colony, which originated from egg masses collected in the vicinity of Goose Lake, Klamath County, Oregon, near the Oregon and California border, was maintained in the laboratory in Corvallis under controlled conditions from the mid-1960s until 1995. Since 1995, the colony has been maintained at the Pacific Forestry Centre (Natural Resources Canada, Canadian Forest Service [NRCan-CFS]) in Victoria, British Columbia, Canada.

TM Biocontrol-1 was produced in vivo. Fifth instars were infected with *O. pseudotsugata* multicapsid nucleopolyhedrovirus (family *Baculoviridae*, genus *Nucleopolyhedrovirus*, *Op*MNPV), reared until death, and the cadavers were harvested and frozen. The USDA–FS processed the first lot of frozen cadavers in a pilot production. Over the next 11 yr (1985–1995), three private companies—Reuter Laboratories, Inc., Espro, Inc., and Crop Genetics, Inc.—were contracted to process the virus-killed larvae and to remove excess insect debris (Hadfield and Magelssen 1995). The finished product was a high-potency wettable powder insecticide with *Op*MNPV as the active ingredient. It could be easily mixed and used with aerial spray equipment under field conditions. The powder was vacuum-sealed in various-sized aluminum foil-lined packages, generally containing between 100 and 1,000 acre-doses (the amount of virus preparation used to treat one acre of *O. pseudotsugata*-infested stands at the registered dose) per package, and placed in long-term storage at −10°C at the facilities of the USDA-FS in Corvallis, OR. The USDA–FS determined the activity or infectivity titer of the processed product on advance samples, provided by the processing companies, by conducting bioassays using the diet surface contamination technique (Martignoni and Iwai 1977) on second instars of *O. pseudotsugata* of the Goose Lake colony. Five different concentrations of the virus were applied to the surface of the artificial diet (Thompson and Peterson 1978) to determine the concentration

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Table 1. Samples of the 10 lots of stored TM Biocontrol-1 bioassayed using third instars of Douglas-fir tussock moth (GL strain) in diet plug experiments at Pacific Forestry Centre in Victoria, British Columbia, Canada, between 1999–2002

Lot no.	Processed by[a]	Package size[b]	g/package	No. packages	Total acre-dose (determined at storage)	TM Biocontrol-1 sample bioassayed[c]	Date stored
1	A	Large	1,200	1	480	1(2×)	1985
2	B	Large	2,150	18	18,000	2(2×) 3 4	1986
3	B	Large	1,830	13	13,000	5 6 7	1986
4a[d]	B	Medium	368	124	24,800	8[g] 10 *12*	1986
4b[d]	B	Large	1,840	33	33,000	9 11 *13*	1986
5a[d]	B	Medium	368	54	10,800	14 16 *18*	1986
5b[d]	B	Large	1,840	26	26,000	15[g] 17[g] *19*[g]	1986
6a	C	Small	47	170	17,000	20[g] 21[g] 22[g]	1989
6b[e]	C	Medium	180	1	383	No sample	1989
7a	C	Small	31.7	483	48,300	23 27 *29*	1990
7b	C	Medium	158.5	100	50,000	24 26 *30*	1990
7c	C	Medium	317	50	50,000	25 28 *31*	1990
8a[f]	D	Medium	202	1	690	32[f]	1991
8b	D	Medium	293	68	68,000	33 34 35	1991
9a[e]	D	Small	49	1	84	No sample	1993
9b	D	Small	58	57	5,700	36 38 40	1993
9c	D	Medium	580	9	9,000	37 39 41	1993
10a	D	Medium	124.7	10	1,000	42 44 *46*	1995
10b	D	Large	1,247	24	24,000	43 45 47	1995
Total		17		1,243	400,237	39	

[a] A, USDA–ARS; B, Reuter Laboratories, Inc.; C, Espro, Inc.; D, Crop Genetics, Inc.
[b] Package (pkg) sizes: small <100 g/pkg; medium, 100–999 g/pkg; and large >1,000 g/pkg.
[c] Bioassaying the samples in italics was deemed unnecessary because the LD_{50} values of the other two samples from the same lot were not significantly different.
[d] Lots 4a and 5a were stored in identical-sized packages (368 g/pkg), as were lots 4b and 5b (1,840 g/pkg).
[e] Lots 6b and 9a had only one package each (383 and 84 acre-doses, respectively); therefore, no samples were taken from these for evaluation of potency after storage.
[f] Sample 32 came from lot 8a and represented only 690 acre-doses; therefore, it was not included in the analysis even though it was bioassayed.
[g] Sample contaminated with CPV.

required to cause 50% mortality in the test insects (median lethal concentration or LC_{50}) (Martignoni and Iwai 1977, 1978).

Small quantities of the products were used experimentally in the field. By 1995, there remained 1,243 vacuum-sealed packages, containing the equivalent of ≈400,000 acre-doses (Table 1) (Hadfield and Magelssen 1995). From these packages, 47 samples were selected for potency testing under a Cooperative Agreement between the USDA–FS and NRCan-CFS. The rest of the registered product is still in storage at −10°C in the USDA–FS facility in Corvallis, OR, for future use to suppress Douglas-fir tussock moth outbreaks. The selection of samples was based on lot number, package size, time in storage, and acre-dose (equivalents remaining in storage) (Table 1). The investigations reported here were conducted to determine whether the stored TM Biocontrol-1 and fresh virus were equally effective against different strains of *O. pseudotsugata* (the laboratory or Goose Lake strain, and different field strains), and whether the potency of the product was affected by 1) time in storage, 2) package size, and 3) the companies processing the virus-killed insects.

Materials and Methods

Bioassay Protocol. Bioassays were conducted to determine the current activity titer (potency) of the stored TM Biocontrol-1 against the Goose Lake strain of *O. pseudotsugata* larvae. Potency was measured as the amount of preparation applied to diet plugs that killed 50% of the test population (median lethal dose or LD_{50}) larvae after 14 and 21 d. At the time of the bioassay, it was not known how soon the larvae would die after ingesting the stored product.

Field-collected or Goose Lake colony *O. pseudotsugata* egg masses were decontaminated using the method described in Thompson and Peterson (1978). The decontaminated eggs were placed in sterile petri dishes (150 by 15 mm) and reared at 25 ± 1°C, 50–60% RH, and a photoperiod of 16:8 (L:D) h. Upon hatching, the larvae were transferred to new sterile petri dishes (100 by 15 mm) and reared in groups of 10 on artificial diet (Thompson and Peterson 1978). Newly molted third instars (<24 h old) were used to standardize the age of the test larvae and were starved for 16–20 h before the bioassays.

Virus Preparation. Twenty milligrams of each TM Biocontrol-1 sample was prepared as a dilute slurry with 20 ml of distilled water. Samples were stirred using a magnetic stirrer for 2 h, and viral samples prepared in triplicate. Serial dilutions were made of the virus, based on the original polyhedral inclusion body (PIB) counts, 1 wk before inoculation, and stored at 4°C until used to infect the larvae in the bioassays. Concentrations approximating the LD_{30}, LD_{35}, LD_{45}, LD_{70}, and LD_{75} values for the virus were chosen, as recommended by Robertson and Preisler (1992), for accurate determination of the LD_{50}. Con-

centrations generally ranged from 16 to 200 PIB per larva.

When TM Biocontrol-1 was initially produced, the USDA–FS determined the PIB concentrations of the samples before storage using the hemocytometer method (Kalmakoff 1980). These counts were available in the original bioassay data provided by R. Magelssen (USDA–FS) (Hadfield and Magelssen 1995). However, as a check on the original counts, we did hemocytometer counts of PIB on five randomly selected samples of the TM Biocontrol-1 samples (sample 2 from lot 2; 20 from lot 6a; 36 and 37 from lot 9b and 9c, respectively; and 43 from lot 10b (Table 1)). The current PIB/ml calculated from these samples were the same as the original PIB counts done at Corvallis; therefore, it was deemed unnecessary to recount the remaining 42 samples sent for bioassay.

As a positive control, a fresh *Op*MNPV virus sample was prepared 1–2 wk before each bioassay by homogenizing 100 virus-killed Goose Lake strain Douglas-fir tussock moth larvae (inoculated with *Op*MNPV from lot 1 while in the third instar) in 5 ml of distilled water. The fresh homogenate was filtered through cheesecloth, centrifuged once at 10 × *g* for 5 min and twice at 7100 × *g* for 20 min on a Hermle Labnet Z383 centrifuge (Labnet International Inc. Division, Woodbridge, NJ), and resuspended in distilled water. A hemocytometer was used to determine the PIB concentration. The same serial dilutions were prepared from both fresh *Op*MNPV and stored samples and stored at 4°C until used in the bioassays.

Inoculation. Bioassays were conducted using the diet plug inoculation technique (Kaupp and Ebling 1990). One microliter either of inoculum or of distilled water in the negative control was added to each small diet plug (3–4 mg) inside each well of a 24-well tissue culture plate (Falcon, BD Biosciences, Franklin Lanes, NJ). Our previous experiments had shown that the diet plugs were large enough to fully absorb the 1 μl of liquid and small enough that a third instar of *O. pseudotsugata* could generally consume it in 24 h.

Five virus concentrations of each TM Biocontrol-1 sample, a fresh *Op*MNPV sample (positive control) and an untreated or negative control (distilled water) were tested in each bioassay. Forty-eight larvae were used for each viral concentration and in the controls. Each bioassay was replicated three times with the replicates conducted on three successive days. Each replicate consisted of 1,488 larvae [five dilutions × 48 larvae × (five virus samples + 1 positive control) + 1 negative control].

After the addition of inoculum to the diet plugs, one newly molted third instar (<24 h old), starved for 24 h, was placed in each well to feed on the treated diet plug. Larvae were held in darkness for 24 h at 25 ± 1°C and 50–60% RH. Only larvae that consumed the entire diet plug were used in the bioassays; they were placed individually in cups (Solo P100, Solo Cup Co., Urbana, IL) with fresh, untreated diet and reared at 25 ± 1°C, 50–60% RH, and a photoperiod of 18:6 (L:D) h.

Because of the high virulence of *Op*MNPV, larvae were reared individually after inoculation to avoid cross-infection. In addition, control and virus-infected insects were reared in separate growth chambers, set at the same rearing conditions, to guard against viral transmission to the control insects. Diet was changed weekly or more often if it dried out. Larvae were reared for 21 d postinoculation, and the mortality was recorded daily. Only larvae that died from nucleopolyhedrovirus (NPV) infection, as determined by gross pathology and verified by microscopic examination, were included in the analysis.

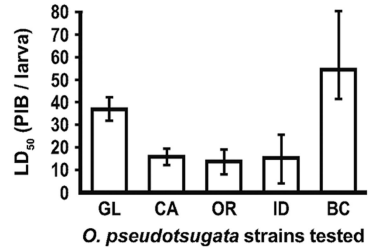

Fig. 1. LD_{50} values of fresh *Op*MNPV in third instars for several different strains of *O. pseudotsugata*. GL, Goose Lake strain; CA, California strain; OR, Oregon strain; ID, Idaho strain; BC, British Columbia strain.

TM Biocontrol-1 Samples Selected for Testing. The 10 lots of TM Biocontrol-1 were stored in a total of 1,243 aluminum foil-lined vacuum-sealed packages ranging in size from 31.7 to 2,150 g/package (Table 1). Of these packages of TM Biocontrol-1 product, 47 samples, each weighing 20 g, were sent to the Pacific Forestry Centre in Victoria, British Columbia, Canada, for testing. The 47 samples, representing 17 different package sizes, were grouped into three weight classes: small (<100 g/pkg), medium (100–999 g/pkg), and large (>1,000 g/pkg).

Of the 47 samples of stored TM Biocontrol-1, at least two of each package size within lots were tested, and more samples (up to six) were bioassayed from lots that had large acre-doses of the product in storage (Table 1). Two of the samples were tested twice for a total of 41 individual bioassay runs over 3 yr. Lots 6b, 8a, and 9a had only one package each (383, 690, and 84 acre-doses, respectively) and were excluded from the analysis because of their small sizes. Of the remaining lots, five had two package sizes (lots 4, 5, 7, 9, and 10), and the LD_{50} values of representative samples of each package size were compared within these lots.

Douglas-fir Tussock Moth Strains Used in Testing. The potency of a fresh sample of *Op*MNPV was compared among four different field strains of Douglas-fir tussock moth (from California [CA], Oregon [OR], Idaho [ID], and British Columbia [BC]), and the Goose Lake laboratory strain to determine whether host strain had an effect on efficacy. Bioassays in year 1 of the testing, using field-collected *O. pseudotsugata*, indicated that geographic origin of the field strain of the *O. pseudotsugata* affected the LD_{50} values (Fig. 1). To eliminate host-caused variability, Goose Lake larvae were chosen for the comparisons of time in storage, possible effects of package size, and possible differences in potency caused by processing by the

different companies. In addition, egg masses could be obtained more readily and reliably from laboratory rearing than from field collections; the laboratory strain is also free of egg parasites and the naturally occurring virus, and the Goose Lake strain also could be produced year round in the laboratory with the appropriate cold storage to break diapause. Furthermore, the original potency data used for registration was obtained from tests conducted with the Goose Lake strain.

Data Analysis. Dosage–mortality curves and LD_{50} values in PIB with associated 95% fiducial limits were calculated using PROC PROBIT analysis (SAS Institute 1996). Each replicate at day 14 and day 21 postinoculation was tested as a separate preparation to determine whether there were any differences between the replicates. LD_{50} values were calculated for each TM Biocontrol-1 sample and the fresh *Op*MNPV sample, and values at day 14 and day 21 were compared to see whether mortality changed over time. LD_{50} values were examined for significant differences (no overlap of the 95% fiducial limits). For larval mortality data, probit lines were compared and differences were considered significant when $P < 0.05$.

For each bioassay, the concentrations chosen to calculate the LD_{50} were based on previous experiments as a best estimate of what was appropriate. However, the samples were not all the same and in rare cases the dilutions chosen gave mortality levels that resulted in wide confidence limits. Because the bioassays are costly and time-consuming, only 41 of the 47 TM Biocontrol-1 samples were bioassayed (two samples were bioassayed twice), and bioassays were not repeated unless the results of at least two of the three samples were significantly different (Table 1). Results from 39 of the bioassays were used in the analysis.

Results and Discussion

Potency of Fresh and Stored TM Biocontrol-1 Samples against Different Field Strains and the Goose Lake Strain. The diet plug bioassay technique is superior to the diet surface contamination method because it eliminates the effects caused by variation in the distribution of PIB on the diet surface or differential feeding rates among the larvae. When the diet surface contamination technique is used, there can be differences in larval feeding rates and the number of PIB consumed, and these also may be affected by interference because of crowding among larvae reared in the same container. There were no significant differences in mortality ($P < 0.05$) among the three replicates or between LD_{50} values at day 14 compared with day 21 for the 39 viral samples tested. Therefore, data of the three replicates were combined, and we used the day 14 data in the analyses.

Challenging different field strains of *O. pseudotsugata* with fresh *Op*MNPV samples showed that there was variation in the susceptibility among the strains of *O. pseudotsugata* from different geographic areas (Fig. 1). The three field strains from the United States (CA, OR, and ID) had similar, relatively low LD_{50} values compared with the BC strain. The LD_{50} value of the BC strain was significantly higher, about three-fold higher with fresh *Op*MNPV, when overlap of the fiducial limits is compared. The LD_{50} values for the Goose Lake strain challenged with fresh *Op*MNPV were intermediate (Fig. 1).

LD_{50} values among the different TM Biocontrol-1 samples tested were similar (data not shown) to those tested with fresh *Op*MNPV sample (Fig. 1) for the CA and ID strains (fiducial limits overlap). There were insufficient numbers of OR larvae for these additional tests. Several studies also have reported that the strain of insects used in bioassays can affect the susceptibility to NPV. Milks (1997) reported a 3.5-fold difference in susceptibility of 12 lines of cabbage looper, *Trichoplusia ni* (Hübner), to the singly embedded nucleopolyhedrovirus (family *Baculoviridae*, genus *Nucleopolyhedrovirus*, *Tn*SNPV). Vail and Tebbets (1990) reported a significantly higher LC_{50} value (2.3- and 3.4-fold) for two of the wild-type populations, but not for the other four populations (two wild-type and two laboratory strains) they tested of the Indianmeal moth, *Plodia interpunctella* (Hübner), to a granulovirus. Reichelderfer and Benton (1974) found a five-fold difference in the LC_{50} values of two strains of *Spodoptera frugiperda* (J. E. Smith) given the same doses of an NPV incorporated into the diet. Skatulla (1987) observed variations of mortalities ranging from 26.1 to 90.0% in gypsy moth, *Lymantria dispar* (L.), larvae from different geographical regions of Europe when the larvae were given the same concentration of an NPV in bioassays. Similarly, Aratake (1973) found wide variation in LC_{50} values among various strains of *Bombyx mori* (L.) challenged with NPV.

Because there is variation in the LD_{50} values of different hosts from different geographic locations, it is necessary to consider the target population when determining the acre-dose for suppression or control projects. If the target field strain of Douglas-fir tussock moth is more susceptible to *Op*MNPV in newly infested areas requiring treatment, it may be possible to use less than the registered dose of TM Biocontrol-1 per acre and still achieve the desired levels of larval mortality and foliage protection. Reduced dosages of TM Biocontrol-1 were tested successfully in BC (where the insect is less susceptible to *Op*MNPV, (Fig. 1), and it was found that the registered dose (2.5×10^{11} PIB/ha) can be reduced by approximately two-thirds and still achieve high larval mortality (95% at full dose, 91% at one-third dose) (Otvos et al. 1987). Perhaps the efficacy of reduced dosages of virus application also should be tested under field conditions in the United States.

Ideally, activity standardization bioassays should be done, preferably using the Goose Lake strain, as the first step in acre-dose determination (Martignoni 1978; D. W. Scott, unpublished data). Each of the 10 production lots of TM Biocontrol-1 has a potency or activity titer that equates to an acre-dose treatment, based on bioassays with the Goose Lake strain, and is expressed as activity units per gram (AU_{GL}/g) (Mar-

Table 2. Comparison of LD_{50} values and potency ratios to fresh *Op*MNPV, of the different lots of TM Biocontrol-1 samples stored for various lengths of time at −10°C at Corvallis, OR

Sample	Processed by[a]	Yr in storage (to 2000)	LD_{50} (PIB)	Upper CL	Lower CL	Potency ratio[b]
Lot 1	A	15	77.06	95.31	65.48	0.48
Lot 2	B	14	49.60	56.29	43.10	0.75
Lot 3	B	14	61.02	67.64	55.19	0.61
Lot 4	B	14	52.05	57.18	47.59	0.72
Lot 5	B	14	45.65	49.76	41.88	0.82
Lot 6	C	11	44.53	49.45	39.71	0.84
Lot 7	C	10	47.63	58.44	37.87	0.78
Lot 8	D	9	113.76	131.12	100.28	0.33
Lot 9	D	7	55.94	61.68	50.92	0.67
Lot 10	D	5	54.04	59.32	49.21	0.69
Fresh *Op*MNPV	n/a	0	37.26	42.87	31.85	1.00

Only data from bioassays done with the Goose Lake strain were used to calculate potency ratios. n/a, not applicable.

[a] A, USDA-ARS; B, Reuter Laboratories, Inc.; C, Espro, Inc.; and D, Crop Genetics, Inc.

[b] Potency ratio is the ratio of the LD_{50} value of the TM Biocontrol-1 samples of that particular lot compared with the LD_{50} value of the fresh *Op*MNPV.

tignoni 1978; D. W. Scott, unpublished data). After determination of potency losses of the various lots of stored product in the laboratory, using the Goose Lake strain, the acre-dose to be used for field application should be calculated from a second bioassay and tested for efficacy in the field. In this second bioassay, *O. pseudotsugata* larvae from the target field population (the population to be treated) and TM Biocontrol-1 from the lot(s) to be applied during that particular suppression project should be used. These two bioassays, ideally, should be done each time a new, separate field population is scheduled for treatment in a new area and whenever a new lot of the stored product is used.

Effects of Time in Storage on the Potency of the Product. A total of 39 TM Biocontrol-1 samples were bioassayed from the 10 lots (Table 1). Because package size of the stored virus product, as expected, did not affect the potency within a lot, bioassay data from samples in a lot were combined and analyzed with SAS PROC PROBIT (SAS Institute 1996) to determine the effects of storage time on each lot. All fresh virus data also were combined in the same manner to give an overall LD_{50} value for the fresh *Op*MNPV samples (Table 2). There were no significant differences (there is an overlap of 95% fiducial limits) among the LD_{50} values for eight of the 10 lots (lots 2–7, 9, and 10). The LD_{50} for lot 1 is significantly higher than all lots except for lot 3 and is significantly lower than lot 8. The LD_{50} for lot 8 is significantly higher than all the other lots. These results suggest that there was a loss of potency of the stored TM Biocontrol-1 over time, but a direct correlation with length of storage is not apparent (Table 2).

Potency Ratios. Relative potency provides a convenient comparison of the differences among samples, and is defined as the ratio of equally effective doses (Finney 1971). For bioassays using the Goose Lake strain, potency ratios were calculated by comparing the overall LD_{50} of all fresh *Op*MNPV samples (potency ratio of 1.0) to the combined LD_{50}s of each of the 10 lots of the stored TM Biocontrol-1. Results indicate that each lot is numerically less potent than a fresh sample of *Op*MNPV. However, seven of the 10 lots retained a potency of ≈0.7 or greater (Table 2), suggesting that they have maintained most of their potency.

When the combined LD_{50} values for each of the lots are compared with the combined LD_{50} value for a fresh sample of *Op*MNPV, there is no overlap of the 95% fiducial limits between the fresh virus and any of the 10 lots. All 10 lots have significantly higher LD_{50} values (confidence limits do not overlap) than the fresh *Op*MNPV (Table 2), indicating that all lots of TM Biocontrol-1 in storage experienced some loss of potency over time. Lots 1, 3, and 8 had the lowest potency ratios (0.5, 0.6 and 0.3, respectively) and had been in storage for 15, 14, and 9 yr, respectively (Table 2). The largest decreases in potency, seen in lots 1 and 8, did not seem to be directly related to time in storage, because lot 8 had only been stored for 9 yr, yet had the greatest loss of potency (ratio of 0.3), and lot 1 with the longest time in storage of 15 yr had the second greatest (0.5) loss of potency. It also should be noted that lots 2, 4, and 5, which also have been in storage for 14 yr, had potency ratios of 0.7, 0.7, and 0.8, respectively. The low potency ratio for lot 8 is anomalous, and we could only speculate on the reason for this occurrence. Lot 8 was the most potent of the 10 lots when the activity of each lot was determined from the advance samples provided by the various companies processing the virus (unpublished data). All lots were stored under the same conditions; therefore, it is presumed the anomaly is not because of storage.

If length of time in storage had directly affected the potency in a linear manner, one would have expected an increase in the LD_{50} (PIB) values with increased time in storage. This was not the case (Table 2). It should be noted the original bioassays, done by the USDA–FS in Corvallis, OR, were conducted before storage with an advance sample provided by the processing company. In our case, the virus used in the bioassays had been in storage for five or more years. Processing the virus-infected dead larvae for the product could have had additional impacts that may obscure the effects that time in storage had on the po-

tency of the stored product. This speculation is based on visual impressions of the coarseness of the lots of the product processed by the different companies, i.e., whether additional grinding was necessary to extract viral DNA from the various lots (Reed et al. 2003).

When TM Biocontrol-1 was first produced, efficacy data for the lots were not compared with a fresh batch of *Op*MNPV before storage, and only one (the advance) sample from each lot was bioassayed for quality control. It is possible that the original lots were not as effective as a fresh sample of *Op*MNPV even before the lots were vacuum-packed for storage. Our comparisons only report the potencies as compared with a fresh sample, and they do not necessarily prove that the decrease in potency of the different lots, i.e., lower infectivity from their original state, is entirely because of storage. Reed et al. (2003) found no evidence of change in viral DNA in any of the lots stored at −10°C. Other, yet undetermined, mechanisms also may be responsible for this loss of potency.

Other studies with *Op*MNPV show similar loss of efficacy over time. Morris (1963) applied *Op*MNPV, stored in aqueous suspension at room temperatures for 7 yr and fresh virus, to individual trees (five) in the field and found that the 7-yr-old polyhedra suspension (stored wet and unpurified) caused only 10% larval mortality compared with 90% mortality caused by the fresh virus material. Martignoni (1978), using the diet surface contamination technique, reported a shelf-life (not defined but presumed to mean the ability to cause infection and an acceptable larval mortality) of 5 yr for *Op*MNPV when the virus was stored in a cool, dry place (the exact temperature was not specified). Similarly, Kaupp and Ebling (1993) conducted a diet plug inoculation bioassay with second instars of white-marked tussock moth, *Orgyia leucostigma* (J. E. Smith), and Virtuss (the same virus as *Op*MNPV but produced in *O. leucostigma*) and found that Virtuss stored for 2 yr at 4°C showed a 46% loss in infectivity, whereas a 96% loss in infectivity was observed after 10 yr in storage at 4°C.

Studies with other insects also have reported decreasing infectivity of virus with time in storage. Cunningham (1970b), using balsam fir, *Abies balsamea* (L.) Mill., foliage dipped in virus suspensions stored for 6 yr at 4°C, found that the pathogenicity of eastern hemlock looper, *Lambdina fiscellaria fiscellaria* (Guenee), NPV was greatly reduced. He attributed this loss to damage of the polyhedra of the virus. Because of such loss of potency in virus samples stored as aqueous suspension, freeze-drying was recommended as the preferred method of storage with no adverse effect on the virus (Cunningham 1970a). Lewis and Rollinson (1978) found a similar decrease with stored *L. dispar* NPV. In their diet contamination bioassays using second instars of gypsy moth, suspensions of NPV retained their potency for 5 yr in refrigeration at 4°C, 2 yr at room temperature, 1 yr as air-dried powder stored at 4°C, and 6 mo as air-dried powder stored at 38°C.

Neilson and Elgee (1960), investigating the effect of storage on virulence of NPV on second and third instars of European spruce sawfly, *Gilpinia hercyniae* (Hartig), reported similar results. Using foliage contaminated with virus suspension, they found that when virus was stored at 4.5°C, loss of potency occurred after 5 yr with the greatest change in effectiveness at 9 yr in storage and total inactivation after 12 yr of storage.

Our study examined the change in potency of freeze-dried virus-killed larvae ground to a fine powder that was stored at the coldest temperature examined to date (−10°C) for such a long time (5–15 yr). Because the samples were in a fine powder form without clumping upon arrival for testing they were assumed to be free of moisture, thus excluding the possibility that the presence of moisture contributed to the loss of potency. Because TM Biocontrol-1 was stored at a much lower temperature than those previously reported, we would expect smaller losses of potency in our samples. Storing at an even lower temperature (−20°C) may further reduce the rate of loss of the potency of stored virus products.

Effects of Package Size on Potency. No virus lot had more than two package sizes (Table 1), and the potential effects of package size on potency were investigated in five of the lots (Fig. 2). LD_{50} values of 22 samples from the five lots of stored TM Biocontrol-1, representing small (four), medium (12), and large (six) package sizes, were compared in 24 different bioassays using the Goose Lake strain. Of the 24 different comparisons of LD_{50} values obtained from small, medium, and large samples, 18 (or 75%) showed no statistically significant differences in the LD_{50} values.

Three samples, 16 (Fig. 2B), 26 (Fig. 2C), and 41 (Fig. 2D) (all from medium package sizes) (Fig. 2), were the only ones that showed significant differences in their LD_{50} values. Because each of these three samples was involved in two comparisons where there were significant differences, these three samples are suspect, i.e., they were outside the general trend and probably do not represent the whole lot, but rather only the sublot from which they were taken (Table 1).

One possible explanation for these differences may be that the ground up freeze-dried virus-infected larvae, which made up these lots of TM Biocontrol-1, were not properly mixed by the companies producing them. If comparisons using these three samples (16, 26, and 41) are excluded from the overall comparisons of the effect of package sizes, then, of the 18 comparisons made among package sizes, none show differences in LD_{50} values (data not shown). This indicates, as expected, that package size did not affect potency of the virus product stored at −10°C.

Differences in LD_{50} and Physical Properties of TM Biocontrol-1 Because of Processing of Virus-Killed Insects by Different Companies. Lot 1 was processed on an experimental basis and was not included in this comparison. Three private companies (Reuter Laboratories, Inc., Espro, Inc., and Crop Genetics, Inc.), were contracted to process the virus-killed Douglas-fir tussock moth larvae in the other nine lots (Table 1). The LD_{50} data and the potency ratios (Table 2) show

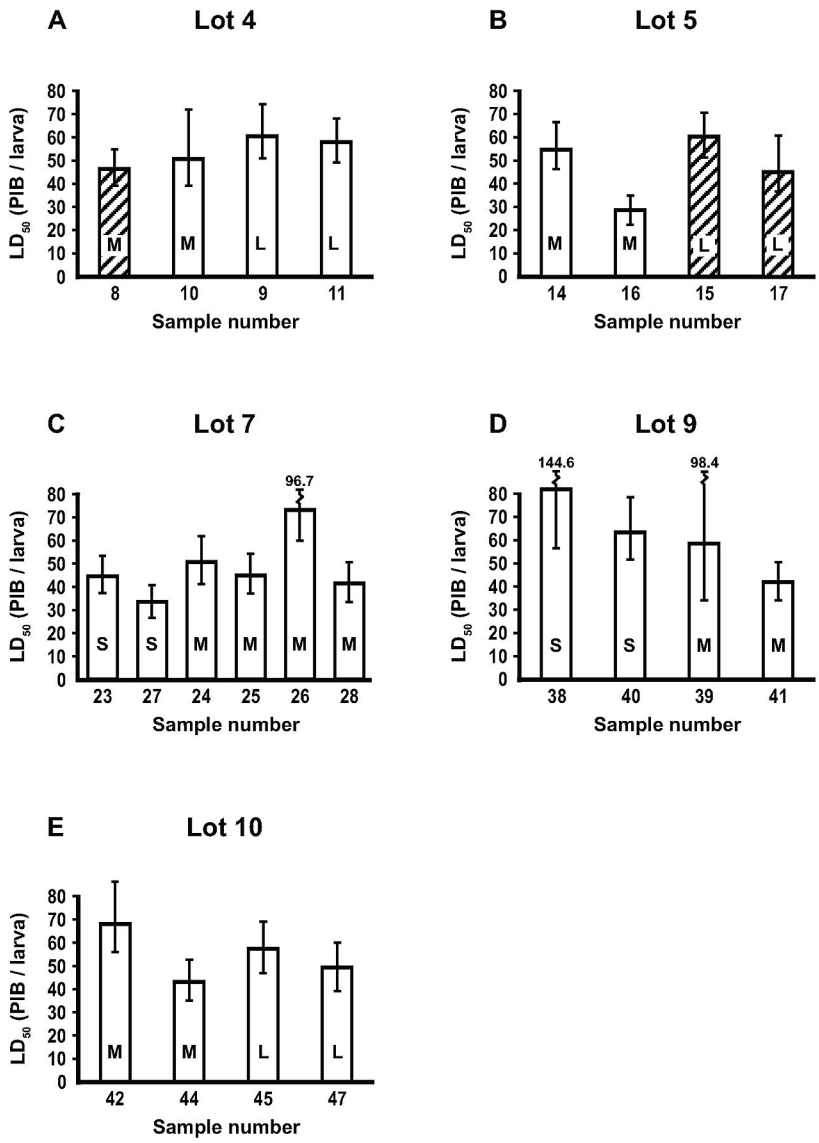

Fig. 2. LD_{50} values for TM Biocontrol-1 samples. S, small package size; M, medium package size; L, large package size. Shading indicates lots that were contaminated with CPV.

no obvious differences in LD_{50} values from the various lots among the three companies processing the virus-killed larvae, with the exception of lot 8, which had about double the LD_{50} values of the other lots. There were also visible differences in the physical properties of the product in some of the different lots (Reed et al. 2003). These differences may have been due, in part, to the processing methods used by the different companies. Unfortunately, the methodology used by two of the three companies to process the virus-killed insects was not available to us.

There seems to be no obvious reason why lot 8, stored for only 9 yr, had the lowest potency, unless it was already less potent at the time of packaging. It was the first lot processed by Crop Genetics, Inc., so this may have had an effect. Generally, there is a learning curve for most activities and processing viral-infected insects into a fine powder is no exception. The other two lots produced by the same company (lots 9 and 10) have much lower LD_{50} values (Table 2). The Government contracts with the processors set standards that had to be met and we can only assume that this was done.

During bioassays of the quality control samples at Corvallis, lot 6 was shown to be contaminated with cypovirus (CPV). This was confirmed by our DNA

analyses of samples from lot 6. In addition, we also found CPV contamination in sublots 4a (sample 8) and 5b (samples 15, 17, and 19). Tanada (1956) reported synergistic effects between NPV and CPV. Our data seem to contradict the synergistic effect reported by Tanada (1956) because of the seven CPV-contaminated samples (8, 15, 17, 19, 20, 21, and 22) (Table 1), none had LD_{50} values that were significantly lower than samples obtained from noncontaminated parts of the same lots. Three of these seven samples are illustrated in Figs. 2A and 2B. Samples 19 and 22 were checked for CPV but not bioassayed, and the LD_{50} for sample 21 is not shown. Likewise, lot 6 did not have a significantly different LD_{50} compared with a majority of the other lots of TM Biocontrol-1 produced (Table 2).

In conclusion, there were significant differences in the susceptibility of Douglas-fir tussock moth larvae reared from different geographic regions to both freshly produced *Op*MNPV and TM Biocontrol-1 infection. There was an ≈30% reduction in potency of TM Biocontrol-1 stored for 5–15 yr at −10°C, but there was no apparent correlation between this decrease in potency and length of storage time. The size of the package in which the product was stored, as expected, did not seem to have an impact on potency. The efficacy of reduced dosages of TM Biocontrol-1 from the currently registered dose should be tested in the field in the United States. It also may be desirable to store the virus product at −20°C; storage at this lower temperature may extend the "shelf-life" of TM Biocontrol-1.

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